

Human Milk Studies

Protein and Non-protein-nitrogen
at the Basle Children's Hospital Milk Bank

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The superiority of human breast milk over other kinds of infant food depends on a series of its properties. Some of the most important of which are supposed to be the easy digestibility owing to its specific composition, the availability of secretion, and its special antibody quality.

Before the first World War, *Bergmann* [2] and *Hotzen* [3] found that undernourishment or inadequate diet on the part of the mother reduced the amount of milk produced. *Klotz* [4] found that when the child was not brought to the breast often enough the milk production decreased. *Czerny* [5] and *Schick* [24] found that when long periods of undernourishment have passed, a true reduction in the secretion results. The increase of milk production of normal women was tried by increasing the diet of these women but the results were doubtful [5]. *Prochazka* [6] was able to increase the milk secretion in women by increasing excretion of Follicular Hormone, which has a hindering effect on the milk secretion. This was accomplished through a raised urinary output with Deriphyllin diuretic suppositories. This increase in milk production has nothing to do with the diet of the women. *Nims et al.* [19, 23] found that women differ in their milk secretion due to hereditary or other reasons, so that it is possible to have a large milk secretion without the suckling stimulation. By experiments on animals and with a woman that was a paraplegic it was possible to show that nerve stimulation plays no rôle in the secretion of milk [21]. In contrary to this was also shown that distraction, by painful stimuli, reduces the amount of milk secretion in the breast of an otherwise normal woman [16, 17]. This experiment was a proof of the animal experiments in the mechanism of lactation [18]. By transplanting the breasts of a mouse on the ears of its sister and allowing the sister to bare young, the transplanted glands also secreted milk showing that location of the glands plays no rôle [22].

From the Veterinary medicine it is a fact that a well nourished cow usually has a very high milk production, but even with good nourishment in a cow, a bad milk producer was never turned into a good milk producer. A good milk producing cow with poor nourishment conditions can have a reduction in milk production [7, 8].

In the qualitative aspect of the milk production during the first World War, a reduction in the protein value was found in human breast milk [2, 4]. Other authors found no changes in the protein value of human breast milk during the war, due to undernourishment [10, 11]. Still others contend that it depends on the region, type of undernourishment, type of diet and time of day [11-13, 5, 24, 2, 3]. When the child is not regularly breast fed there is a tendency for the secretory glands to stop producing milk and in this way milk secretion is reduced [4, 13].

In Sicily the Kwashiorkor cases of breast fed children were believed due to bacteria rather than to protein deficiency since the breast milks tested were found to contain the equivalent amount of protein to that of women in Northern Italy where no Kwashiorkor was present [14-15].

The nitrogen composition and nutritional value of human breast milk is generally appraised in terms of total protein. The value of the total protein in human breast milk expressed in grams percent is 1.0-1.5 [20, 25-32, 53, 56].

The total protein of human breast milk depends on the time post partum. Early breast milk, colostrum, up to 15 days, has a very high content of protein. After the fifteenth day the amount of protein begins to fall although there are very large fluctuations in the amount of total protein between different women and in the same women [19]. *Macy, I. G. et al.* [23] showed a variation of total protein up to 20 percent at intervals of 4 hours in the breast milk of the same women. After 14 months of breast

feeding the protein value was found to be below the average of 1.0 gram percent [20]. The secretion of milk between the two breasts of one woman may vary to a large degree, but the protein content of the milk of each breast remains the same [21]. The human breast milk protein can vary, depending on how long it has remained in the glands of the breast and due to bacterial breakdown [22]. *Møllgaard* [8] and *Liepmann* [9] showed that in undernourishment conditions, if the protein part of the diet was held up, the milk secretion was at a normal level, but when the protein part of the diet was reduced, the milk secretion was also reduced, thus showing, in contrary to other authors [2-3, 5, 24], that a specific type of undernourishment does play a rôle in the secretion of human breast milk.

The non-protein-nitrogen is often included when the protein or nitrogen is determined but is usually disregarded. This small fraction of nitrogen can be significant in physiology of milk secretion [33], and in the nourishment of the infant [34] even when only traces are found. Some workers showed indications that these components reflect the gross composition of dietary changes [35-36].

Non-protein-nitrogen may be derived from two sources. The first is filtered directly from the blood and has the same concentration as the blood [42, 45-46]. The non-protein-nitrogen in breast milk may be at the blood concentration level or depending on its physiological value in the breast milk, it may be higher or lower [50]. The second source is the abnormalities in the milk or glands due to bacteria [36-41].

It was however found that the non-protein-nitrogen in human breast milk in the beginning of lactation was much higher than the blood levels and very irregular [38]. The amount of non-protein-nitrogen in human breast milk varies as much as 50 percent in 4 hour interval analysis in the same women, and again 50 percent variation in day to day samples of breast milk from the same women. The variation climbed to over 100 percent in month to month samples of breast milk from the same women [22].

Indications were that no true value could be set, as every slight upset seemed to change the value of the non-protein-nitrogen. It was thought at one time that this change in the non-protein-nitrogen of the breast milk could be used as an index for bodily disturbances, but the work was not carried further [50]. It has also been seen that infection of the glands or bacterial contamination can also increase the amount of non-protein-nitrogen in breast milk [36, 38-41, 51, 62].

Many different points of view have been written or discussed on the subject of human breast milk, yet all authors believe that human breast milk is the best nourishment for the newborn. Even when the total protein is low and/or the non-protein-nitrogen is high.

At the Basle Children's Hospital it is believed that human breast milk is the best means of nourishment for the newborn. Since at the hospital it is not possible to obtain breast milk in large enough quantities to be used for all the infants, it is used for prematurely born, children with nourishment disturbances such as dyspepsia, atrophy, dystrophy, pyloric stenosis, and coeliac disease, and children that have been found allergic to animal protein (cow's milk and cow milk products).

Breast milk is bought by the Children's Hospital from women that have born a child and have an excess of milk. Before the breast milk is taken from these women, they must be tested for tuberculosis and syphilis. Having passed these examinations the breast milk is brought to the hospital's milk kitchen in 250 ccm. bottles. The milk is collected by the women for 24 hours at home and brought into the hospital once a day. The bottles are supplied by the hospital and each one contains 2.5 ccm. of Didrothenat (one gram Didrothenat in 100 ccm. of a Sodium Chloride solution). Didrothenat is a mixture of 20 percent Dihydrostreptomycine pantothenat and 80 percent Dihydrostreptomycine sulfate. At the milk kitchen the breast milks, at random are further tested bycterialogically and for falsification by addition of cow's milk or water. When the milk has passed these examinations it is prepared for use by sterilization and held in a frigidare until needed.

Determinations

At the Basle Children's Hospital the nitrogen was determined on a variety of different human breast milks. The determination on these breast milks were completed within one hour after arrival in the milk kitchen. The total nitrogen and non-protein-nitrogen were calculated.

The Kjeldahl method of nitrogen determination was used.

Principle: Nitrogen is determined in a portion of the milk by a Macro-Kjeldahl method, using an acid solution for the digestion which liberates ammonia, this in turn is converted to ammonium sulfate, the ammonia in the sulfate is converted into ammonium hydroxide by the base, sodium hydroxide. The ammonium part of the ammonium hydroxide reacts with a known standard of sulfuric acid and is titrated against a known standard of sodium hydroxide with methyl red as an indicator.

Method: For total Nitrogen take 0.5 ccm. milk and 4.5 ccm. Aqua dest., from this use one ccm. in a Kjeldahl flask with one ccm. copper sulfate and two ccm. of concentrated sulfuric acid, and two glass beads. Digest for at least 45 minutes. This is then distilled with an excess of $33\frac{1}{3}$ percent sodium hydroxide, into 5 ccm. of a 0.02 N sulfuric acid solution. Then titrated against a 0.02 N sodium hydroxide solution with methyl red as indicator. For non-protein-nitrogen take 2 ccm. milk and 8 ccm. Aqua dest. and 10 ccm. of 10 percent Trichloroacetic acid, then filter until a clear solution results. Take 5 ccm. of this filtrate in a Kjeldahl flask with 1 ccm. of copper sulfate and 2 ccm. of concentrated sulfuric acid, and two glass beads. Digest for at least 45 minutes. This is distilled and titrated the same way as for the total nitrogen.

The difference between the two determinations times 6.32 (constant) is equal to the total protein in human breast milk.

Example of the calculation:

For total protein:

Controlled titration 5.2

Factor 5 : 5.2 = 0.96

Total nitrogen titration = $4.125 \times 0.96 = 3.96$

$5 - 3.96 = 1.04$, $1.04 \times 0.28 = 0.2912$ g. % = 291.2 mg. %

291.2 mg. % less non-protein-nitrogen 44.2 mg. % = 247 mg. %

$247 \text{ mg. \%} \times 6.32 = 1561.04 \text{ mg. \%} = 1.56 \text{ g. \%} = \text{Total Protein.}$

Example of calculation of Non-protein-nitrogen:

Controlled titration = 5.1

Factor 5 : 5.1 = 0.98

Non-protein-nitrogen titration = $4.5 \times 0.98 = 4.41$

$5 - 4.41 = 0.59$, $0.59 \times 0.28 \times 200$ or $0.59 \times 56 = 33.04$ mg. %.

The following nitrogen determinations were done:

- (1) A variety of different breast milks with Didrothenat.
- (2) Breast milk taken each day from the same women for six consecutive days with Didrothenat.
- (3) Breast milks taken one time from women less than 14 days post partum and kept in a refrigerator, without any artificial conservation. The determinations were carried out at four day intervals on the same milks.
- (4) Breast milks under artificial conservation (Didrothenat and sterilization) and kept in a refrigerator for ten months.

(1) A variety of different breast milks with Didrothenat.

These nitrogen determinations were done to see if the amount of protein and non-protein-nitrogen are within normal limits and to find out if the method of collection the human breast milk is at optimum. A total of thirty-five different breast milks were examined according to the method explained above.

Case	Non-protein-nitrogen in mg. percent	Total Protein in grams percent
1.	64.40	0.99
2.	60.50	1.42
3.	65.60	1.32
4.	77.70	1.22
5.	61.00	0.55
6.	60.50	0.83
7.	85.10	1.11
8.	79.00	0.99
9.	58.20	0.64
10.	49.80	0.64
11.	59.40	1.76
12.	68.30	1.48
13.	51.50	0.77
14.	51.50	1.32
15.	60.50	1.42
16.	66.50	1.37
17.	52.10	0.93
18.	62.20	1.53
19.	48.20	1.22
20.	77.90	1.53
21.	50.40	1.12
22.	77.90	1.22
23.	58.30	0.89
24.	60.50	1.22
25.	44.80	0.89
26.	62.20	0.57
27.	67.20	1.32
28.	73.90	1.01
29.	68.90	1.38
30.	87.40	1.01
31.	60.50	1.02
32.	76.80	1.42
33.	54.30	0.93
34.	63.40	1.22
35.	51.50	1.00

In the determination of non-protein-nitrogen a range of 42.80 mg. % to 87.40 mg. % was found, with an average value of 63.30 mg. %.

In the determination of the total protein a range of 0.55 gram % to 1.76 gram % was found, with an average value of 1.13 grams %.

Comment: This set of nitrogen determinations show a large range in the non-protein-nitrogen and in the total protein of the different breast milks. The inference from this is that the conditions under which the breast milk is kept and collected before being brought to the hospital are not always those to be considered optimum. Even though the milk is treated with Didrothenat, if the milk remains over twenty-four hours without being sterilized there is a greater chance for bacterial changes to take place [73].

There appears to be no conclusive evidence to show a relationship exists between the amount of non-protein-nitrogen and the amount of total protein [22, 50, 73].

In the average of these values the total protein is within the range of what is considered the normal value [26, 30-32], but the average of the non-protein-nitrogen is somewhat higher in relationship to what is considered the normal value [26, 29, 32].

(2) Breast milk taken each day from the same women for six consecutive days: This set of nitrogen values was performed to determine if women themselves vary in their daily nitrogen output (protein and non-protein-nitrogen) in breast milk. These women were all six weeks or more post partum.

Non-protein-nitrogen in mg. percent		Total Protein in grams percent	
range	average	range	average
Mrs. A. (47.00-76.20)	60.90	(0.83-0.98)	0.89
Mrs. B. (44.80-64.40)	52.90	(0.88-1.52)	1.17
Mrs. C. (54.30-57.70)	56.70	(0.85-1.42)	1.08
Mrs. D. (53.70-75.60)	63.50	(0.73-1.60)	1.08

Comment: In each of the preceeding cases, the total protein and the non-protein-nitrogen did vary to some degree but in many instances the degree of variation was not very great. The observations made were that many women tend to remain very constant in their total protein and non-protein-nitrogen output in breast milk, while other women differ daily [19, 23]. The daily diet of these women was not controlled and this could perhaps play a certain role in the nitrogen changes [2, 4, 62].

	Case	Non-protein-nitrogen in mg. percent	Total Protein in grams percent
First day:	1.	79.50	1.46
	2.	76.20	1.81
	3.	72.80	1.31
	4.	44.80	1.41
	5.	52.00	1.21
	6.	79.50	1.36
	7.	72.70	1.47
	8.	71.10	1.50
	9.	84.60	1.74
Fourth day:	1.	90.70	1.22
	2.	85.70	1.32
	3.	79.00	1.17
	4.	67.20	1.37
	5.	70.60	1.13
	6.	95.20	1.14
	7.	78.10	1.23
	8.	77.80	1.33
	9.	84.80	1.68
Eight day:	1.	96.90	1.06
	2.	91.80	1.23
	3.	86.00	1.08
	4.	76.60	1.25
	5.	73.40	1.08
	6.	95.30	1.10
	7.	79.20	1.19
	8.	82.90	1.12
	9.	89.20	1.53

(3) Breast milks taken one time from women less than 14 days post partum and kept in a refrigerator, without any artificial conservation. The determinations were carried out at four day intervals on the same milks. This set of nitrogen determinations was done to find out what changes result when breast milk is held without artificial conservation methods.

Comment: This set of nitrogen determinations show that if breast milk is left standing, even in a refrigerator, without artificial conservation method, the bacterial and/or proteolytic enzymes break down the protein and increase the non-protein-nitrogen content of the breast milk. The existence of these enzymes in raw breast milk has been proven by *Erickson et al.* [50], *Kluge* [62], and *Von Sydow* [73].

(4) Breast milks under artificial conservation (Didrothenat and sterilization) and kept in a refrigerator for ten months. This set of nitrogen determinations was done to see if the conservation methods of human breast milk, at the Basle Children's Hospital, is adequate. These breast milks are pooled milks from different women that brought their breast milks on the same day.

The examination of these breast milks was done on Dec. 13, 1958.

Date	Non-protein-nitrogen in mg. percent	Total Protein in grams percent
Feb. 13, 1958	45.36	0.98
Feb. 15, 1958	54.88	0.94
Feb. 18, 1958	52.64	0.92
Feb. 19, 1958	46.48	0.93
Feb. 22, 1958	56.00	0.91
Feb. 24, 1958	54.32	0.87

Comment: These determinations show that the artificial conservation methods at the Basle Children's Hospital preserve the protein value of the breast milk and the non-protein-nitrogen is not increased, even after ten months. Thus observations show that if human breast milk is treated properly with artificial conservation methods, it is possible to hold this breast milk over long periods of time.

Comment

The nitrogen value of the different milks examined have a very large range and this could be due to various factors. Some factors upon which the nitrogen values of breast milk may depend, which were not controlled, were, the diet, which many authors [2, 3, 6, 23] say can change the nitrogen values, while other authors [11-13] contend that it plays no rôle. The time of day (morning or evening). In the evening the non-protein-nitrogen is higher and the protein value lowest [50]. Stages of post partum (early or late) early post partum shows a high protein and in the later stages the protein value falls [19]. When a woman has emotional upsets the nitrogen value becomes very irregular [22].

Through the different determinations of nitrogen done on breast milk it may be deduced that the uncontrolled factors play a minor rôle in the protein contents of breast milk, since the average of the results are at the normal levels.

The non-protein-nitrogen which was at a higher level then the normal values could be due to any of the above factors, or may be due to bacterial breakdown and proteolytic enzymes. According to *Von Sydow* [73] bacterial changes take place even though the milk contains Didrothenat (Streptomycine). The third set of nitrogen determinations, using raw breast milk (no artificial conservation), shows that there are nitrogen changes in the milk when it is held over a period of time. These changes are probably due to bacteria and proteolytic enzymes. Since the first set of nitrogen determinations had

Didrothenat in them, we may conclude that a proteolytic enzyme system did in some way increase the non-protein-nitrogen.

With the breast milk under artificial conservation and held for ten months, the non-protein-nitrogen was lower than the non-protein-nitrogen in the raw breast milk (that milk not sterilized). It is interesting to note that with 10% Trichloroacetic acid the sterilized milk was much more easily filtered to a clear solution than the unsterilized milk. Since the filtration of the raw milk was more difficult than the sterilized milk there exists the possibility that traces of protein remain causing an increased non-protein-nitrogen. The possibility also exists that an enzyme system was blocked and allowed the filtration to pass without difficulty. The existence of these enzymes in raw milk was proven by *Erickson* [50] and *Kluge* [62].

Conclusions

The conclusions we have drawn from these nitrogen determinations are: (1) The method of collecting the milk, although not under optimal conditions, is still good enough to continue buying the breast milk. (2) The method of artificial conservation carried on at the Basle Children's Hospital is adequate for the preservation of the protein value of the breast milk.

Summary

(1) With the Kjeldahl method of nitrogen determination in a variety of different breast milks, the range of total protein was large but the average of 1.13 grams percent was at the normal level. The range of non-protein-nitrogen was large with a high average of 63.30 mg. percent.

(2) With breast milk taken from the same women on consecutive days, the observations were that some of the women have large variations in daily nitrogen content (over 100 percent) while others tend to remain constant (under 20 percent variation).

(3) Breast milk taken once before the 14th day post partum, when placed in a refrigerator without any artificial conservation method, showed a definite increase in the non-protein-nitrogen and a decrease in the total protein.

(4) Breast milk under artificial conservation methods, carried out at the Basle Children's Hospital, and held for ten months in a refrigerator showed a definite preservation of protein and no increase on non-protein-nitrogen.

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